Combination of PI3K/Akt Pathway Inhibition and Plk1 Depletion Can Enhance Chemosensitivity to Gemcitabine in Pancreatic Carcinoma

Yonghuan Mao[*], Ling Xi[†], Quan Li[‡], Sheng Wang[‡], Zeling Cai[‡], Xinhua Zhang[§] and Chunzhao Yu[‡]

[*]Department of General Surgery, Nanjing Drum Tower Hospital, The Affiliated Hospital of Nanjing University Medical School, 210028 Nanjing, China; [†]Department of Gerontology, Jiangsu Province Official Hospital, 210029 Nanjing, China; [‡]Department of General Surgery, the Second Affiliated Hospital of Nanjing Medical University, 210029 Nanjing, China; [§]Department of Pediatric Medicine, Jiangsu Province Hospital, 210029 Nanjing, China

Abstract

The prognosis of pancreatic cancer (PC) remains pessimistic because of the difficulty in early diagnosis as well as the little advance in chemotherapy. Although being the first-line chemotherapy drug for PC at present, gemcitabine still has some disadvantages, such as low drug sensitivity and significant side effects. Thus, how to further improve the sensitivity of PC cells to gemcitabine is still a difficult subject in the field of pancreatic cancer-treatment. Polo-like kinase 1 (Plk1) is closely related to poor outcome in many malignant tumors and its high expression is linked to chemoresistance in PC. As a downstream gene activated by PI3K/Akt signal pathway, we assumed that the targeted depletion of Plk1 could contribute to the chemosensitization induced by synergistic drug interaction of PI3K inhibitor LY294002 together with gemcitabine. To analyze effect of Plk1 in chemotherapy, we constructed two recombinant adenoviral vectors which carry enhanced green fluorescent protein (rAd-EGFP) and Plk1-shRNA (rAd-shPlk1), respectively. Both inhibition of PI3K/Akt signal pathway through PI3K inhibitor LY294002 and targeted depletion of Plk1 via recombinant adenoviral shRNA can cause chemosensitization, and the targeted depletion of Plk1 can enhance the chemosensitization of LY294002. Thus, the gene therapy like targeted depletion of Plk1 may create new perspectives for chemosensitization of PC.

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Introduction

In 2017, pancreatic cancer (PC) represents 3.0% of all new cancer cases in the United States [1]. Compared to other cancers, PC is relatively rare. However, it is more common with increasing age and has an aggressive behavior with poor prognosis, resulting in an estimated 44,330 deaths in 2017 and making it the third leading cause of cancer death in the United States [1]. Because of its high frequency of chemoresistance, PC is relatively insensitive to conventional chemotherapy. Gemcitabine was recommended as the first first-line drug for chemotherapy of PC and chemotherapy using gemcitabine alone was the standard for about a decade, as a number of trials testing it in combination with other drugs failed to demonstrate significantly better outcomes [2–4]. Hence, how to further improve the sensitivity of PC cells to gemcitabine will provide clues for new targeted therapies.

Polo-like kinase 1(Plk1), a serine/threonine kinase that plays an essential role in cell mitosis, spindle assemble, DNA damage and so on,

is a member of polo-like kinases family [5,6]. It is an early trigger for G2/M transition and localizes to centrosomes during interphase. Plk1 is closely related with the occurrence of tumor development. Compared to normal tissues, it is over-expressed in a broad range of tumors, such as ovarian carcinoma, colorectal carcinoma, prostate cancer, skin cancer, and others [7–10], and has been implicated in tumorigenesis and

progression [11,12]. Overexpression of Plk1 can inhibit the activity of p53 through phosphorylation p53, which cause the apoptosis process failure, then cancerous cells survival which bring out the occurrence of cancer eventually [11,13]. As reported, Plk1 is over-expressed in PC, invasive pancreatic adenocarcinomas were Plk1 positive in 47.7% of cases [14], which means Plk1 overexpression is likely to be related to biological behavior of PC. Therefore, targeting Plk1 in developing small molecule inhibitors as anti-cancer drugs becomes a hotspot in the recent years. Such as Plk1 inhibitor BI2536 has been evaluated for patients with various cancers in clinical trials [15–18].

The deregulation of PI3K/Akt pathway has been confirmed that plays an crucial role in human cancers including PC [19,20]. PI3K/Akt pathway includes a series of cascade: PI3K generation PI3P through phosphorylation PI2P, and PI3P combination with the N-terminal of Akt, then activation of Akt, which activate or inhibit its downstream substrates, such as mTOR, Caspase-9, Bad, etc. [21]. Akt, a serine/threonine kinase which plays a core role in the PI3K/Akt signal pathway. Meanwhile, Akt contributes to cell plasticity in pancreas as a regulator and its overexpression has been proved to be a common phenomenon in PC [22–24]. Until now, several Akt inhibitors have been evaluated in clinical trials [25,26]. Hence, the PI3K/Akt pathway plays a crucial role in the development of PC, and it can be a potential therapeutic target for PC.

Cell cycle is crucial for proliferation, differentiation, and growth both in normal cells and tumor cells. Hence, the factors affecting cell cycle can be used as a potential target of tumor therapy. Whether PI3K/Akt signaling pathway or Plk1 in cell cycle is the indispensable factors, keep unclear. It is reported PI3K/Akt-dependent phosphorylation of Plk1-Ser99 is required for metaphase-anaphase transition, and Plk1-dependent phosphorylation of IRS2-S556 inhibits mitotic exit through reducing Akt activity [27,28]. Yu et al. showed that up-regulation of Plk1 were related to chemoresistance of PC, and Kim et al. found that inhibited PI3K/Akt pathway could increase the chemosensitivity of PC to gemcitabine [29,30]. However, the mechanism of cell apoptosis induced by PI3K/Akt pathway and Plk1 still remains unclear. In current study, we combined the inhibition of PI3K/Akt pathway with down-regulation of Plk1 and observed that the chemosensitivity of PC to gemcitabine further increased. In addition, LY294002 and BI2536 (an inhibitor of Plk1), together with gemcitabine significantly suppressed the growth of xenografts in nude mice model, which made PI3K/Akt pathway inhibition as well as Plk1 down-regulation become a potential target to increase the chemosensitivity of PC.

Materials and Methods

Cell Culture and Reagents

PC cell lines were obtained from ATCC and were maintained in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum (FBS) (BxPC-3 and PANC-1), in RPMI 1640 supplemented with 10% FBS (AsPC-1). Gemcitabine was purchased from Sigma (Sigma-Aldrich Co. LLC, USA), it was dissolved in 100% dimethyl sulfoxide (DMSO, Muskegon, MI, USA) to a stock concentration of 10 mM and stored at –20 °C. The PI3K inhibitor LY294002 was purchased from CST (Beverly, MA, USA). BI2536 (Plk1 inhibitor) was gained from Selleck (Houston, USA), it was dissolved in DMSO and stored at –80 °C. The following antibodies were purchased for Western blot: Plk1, p-Akt, Akt (Cell Signaling

Technology, MA, USA); Bcl-2, BAX (Abcam, UK); and glyceraldehyde 3-phosphate dehydrogenase (GAPDH, Santa Cruz, CA, USA). Plk1 and Akt antibody for immunohistochemistry (IHC) was gained from Abcam (Abcam, UK).

Immunohistochemistry (IHC) and Clinical Specimens

The human pancreatic tissues and related clinical data were purchased from Xian Ailina Biotechnology Co. Ltd. (Xian Ailina Biotechnology Co. Ltd., China). Paraffin-embedded sections of human pancreatic tissues and mouse xenografts were subjected to specific antibodies for Plk1, Akt, Cleaved caspase 3, or isotype-matched controls at appropriate dilutions. IHC staining in human tissues were scored independently by two pathologists, by evaluating a semiquantitative immunoreactivity score (IRS) as described [31]. Then, tissues with IRS 0–5 and IRS 6–9 were defined as low and high expression of Plk1, respectively.

Construction of Recombinant Adenoviral rAd-EGFP and rAd-Plk1-shRNAs (rAd-shPlk1)

Based on gene sequence (GenBank: NM_005030), four shRNAs targeting different regions of the Plk1 transcript were synthesized with the vector pYr-1.1 (hU6/EGFP/Neo) (Changsha Yingrun Biotechnology Co. Ltd., China). After package and screen, both rAd-Plk1-shRNA2 and rAd-Plk1-shRNA4 worked effectively, especially rAd-Plk1-shRNA4 so that the latter was finally used for the following experiment and was set as rAd-shPlk1. The empty vector rAd-EGFP was constructed as an experimental control.

Western Blotting Analysis

Cells were lysed in RIPA Lysis Buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM PMSF) and resolved by SDS-PAGE. Samples were analyzed as described [32]. Phospho-specific antibody to Akt(S473) was detected to determine the level of activated protein, with antibody recognizing total Akt to control for total protein expression. Antibody to Plk1 was used to monitor total protein expression. Antibodies for BAX and Bcl2 were used to monitor apoptosis. Antibody for GAPDH was used to verify equivalent loading of total cellular protein.

RNA Isolation and Quantitative Real-Time PCR

The mRNAs were extracted with Trizol reagent (Life Technologies, Ltd). The cDNAs were prepared with Superscript III (Life Technologies, Ltd) Takara Kit (Dalian, China)as indicated by the manufacturer. The cDNAs were amplified by PCR in an iQ5 Multicolor Real Time Detector System (Bio-Rad) with the fluorescent double-stranded DNA binding dye SYBR Green (Bio-Rad). The relative amounts of gene expression were calculated with GAPDH expression as an internal standard (calibrator). Primers used for the Akt gene: forward primer 5′-TCACCATCA CACCACCTG AC-3′ and reverse primer 5′-CTCAAATGCACCCGAGAA AT-3′. Primers used for the Plk1 gene: forward 5′-ACC AGC ACG TCG TAG GAT TC-3′ and reverse 5′-ATA ACT CGG TTT CGG TGC AG-3′. Primers used for the GAPDH gene were 5′-AAC GGA TTT GGT CGT ATT GG-3′ (forward) and 5′-GGA TCT CGC TCC TGG AAG AT-3′ (reverse) (Invitrogen, USA). The results, expressed as N-fold differences in target gene expression, were determined as follows: N *target = 2 (Ct sample Ct calibrator).

Apoptosis Detection by Flow Cytometry (FCM)

Apoptosis was detected by flow cytometry. The cells were incubated with gemcitabine for 48 h, LY294002 for 48 h or recombinant adenovirus for 24 h, 48 h, 72 h and 96 h; then washed twice with ice-cold PBS and resuspended in 1× Binding Buffer(BD Pharmingen, USA) at a concentration of 1× 105 cells/ml. Added 5 μl of APC Annexin V(BD Pharmingen, USA) and 5 μl of 7-AAD(BD Pharmingen, USA).Then, samples were analyzed by flow cytometer (BD FACSCalibur equipped with CellQuest Pro).

Determination of IC50

To determine the half maximal inhibitory concentration (IC50), $1–2 × 10^3$ cells were seeded in 96-well plates and treated with concentrations of gemcitabine from 0 μM to 20 μM. After 48 h of treatment, cell proliferation was analyzed with CCK8 and read at an absorbance of 450 nm. IC50 values were calculated with CalcuSyn or CompuSyn software.

PC Cell Line Xenografts

Female athymic nude mice (nu/nu, 6 to 8 weeks of age, gained from Chinese Academy of Sciences, Shanghai, China) were maintained under pathogen-free conditions in micro isolator cages. Animal procedures were performed in accordance with the rules set forth in the NIH Guide For The Care And Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee at Merck. All animals received food and water ad libitum. BxPC-3 cells $(1 × 10^7/120$ μl in PBS: Matrigel = 1:1) were injected s.c. into the right flank region of the mice. Tumors were allowed to reach $80–120$ mm^3 before randomization to treatment groups. For continuous dosing studies, gemcitabine was administered intraperitoneally (i.p.) at 100 mg per kilogram body weight (mpk) once daily (qd) for 14 to 21 days, LY294002 (25 mg/kg) and BI2536 (30 mg/kg) were administered i.p. twice a week. Mice in control group received 1%DMSO on the same schedules that gemcitabine, LY294002 and BI2536 were administered. Six mice were in each treatment group. Tumor volumes were measured twice weekly using calipers and calculated by the formula (length × width^2)/2. Animal body weights were measured on the same days twice weekly. Statistically significant differences between the treatment groups were determined by the Student's *t* test and 2-way ANOVA using GraphPad Prism. Six hours after their last dose, mice were euthanized by carbon dioxide inhalation. The tumors were then removed and cut into two pieces. One piece was frozen in liquid nitrogen while the second piece was fixed in 10% formalin for future analysis.

Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling (TUNEL) Assay

Cut the paraffin blocks into slices with 4 μm thickness. The TUNEL Brighted Apoptosis Detection Kit, POD (Vazyme, Nanjing, China) was used to detect cellular apoptosis in tumor tissues according to the instructions.

Statistical Analysis

Data were expressed as mean ± SD unless otherwise specified and each group was statistically analyzed using t-test or ANOVA. All statistical analyses were performed using GraphPad Prism 5.0 and SPSS 20.0 software. Each experiment was performed three times. Differences were considered statistically significant at $P < .05$.

Results

Plk1 is Overexpressed in Human Pancreatic Carcinoma Versus Normal Tissue

We purchased pancreatic tissue microarray (including 59 cases of normal pancreas and 88 cases of PC), and IHC staining was used to detect the expression of Plk1. The staining showed that 83% of normal tissues were Plk1 with low expression (IRS 0–5), 17% of normal tissues were Plk1 with high expression (IRS 6–12); whereas 66% of PC tissues were low expression (IRS 0–5), 34% of PC tissues were high expression (IRS 6–12). The above results mean that the expression of Plk1 in PC tissues was higher than that of the normal pancreatic tissues ($P = .022$) (Figure 1, *A* and *B* and Table 1).

Next, we analyzed the expression of Plk1 in PC tissues and the clinical characteristics of these patients to explore their relationship. The study showed that the expression level of Plk1 in PC tissues has no significantly difference among gender, age or clinical stage ($P > .05$). Nevertheless, Plk1 expression level was correlated with pathologic grade. Pathologic grade I-II with 74% low expression and 26% high expression of Plk1; Pathologic grade III-IV with 48% low expression and 52% high expression of Plk1. These analysis result imply that more PC tissues with high expression of Plk1 in Pathologic grade III-IV than those in Pathologic grade I-II ($P = .019$) (Figure 1*C* and Table 2).

Targeted Depletion of Plk1 via Recombinant Adenoviral shRNA Causes Apoptosis in PC Cells

To further explore the role of Plk1 in PC cells, we constructed the recombinant adenoviral to depletion of Plk1 (rAd-shPlk1). QRT-PCR and Western blotting analysis showed that, under the condition of the same amount of the total sample, GAPDH strips were basically same when we used GAPDH as internal control, while the Plk1 strips were significantly different, the Plk1 mRNA and protein relative quantity in rAd-shPlk1 group was markedly lower than those in the rAd-EGFP groups ($P < .001$) and control groups ($P < .01$) (Figure 2, *A* and *B*). From our data above we believed that both Plk1 mRNA and protein levels were significantly down-regulated by rAd-shPlk1 in PC cells. We used Western blotting to detect apoptosis-related proteins Bcl-2 and BAX levels after down-regulation of Plk1 expression. In result, we found that Bcl-2 was down-regulated and BAX was up-regulated, which means depletion of Plk1 could induce apoptosis in PC cells (Figure 2*B*). Then, we examined the cell apoptosis of each group by flow cytometry. As shown in Figure 3*E*, we checked the apoptosis rates of the control group, the rAd-EGFP group and the rAd-shPlk1 group at 24 h, 48 h, 72 h and 96 h, respectively. Obviously, during the first 24 h, apoptosis rates among these three groups showed no significant difference ($P > .05$); while after 48 h, the rAd-shPlk1 group had the highest cell apoptosis rate among them ($P < .01$) and comparing to the control group and rAd-EGFP group, the viable cell number of the rAd-shPlk1 group was significantly lower than that of the rAd-EGFP group at each time point (Figure 3, *C–E*). Hence, we inferred that rAd-shPlk1 could induce the apoptosis of PC cells.

Gemcitabine-Sensitivity in PC is Closely Related to PI3K/Akt Signal Pathway

As mentioned earlier, the PI3K/Akt signal pathway are closely related to cell cycle, proliferation, differentiation, apoptosis and so on, and the development and drug-resistance of most malignant tumors
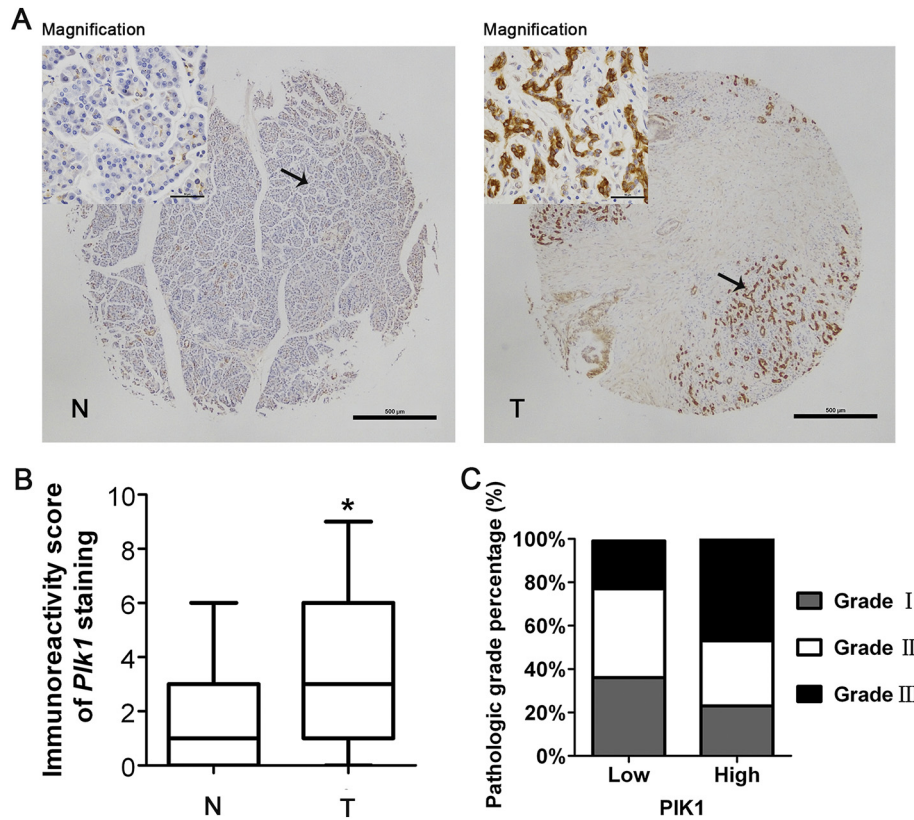
Figure 1. Plk1 is over-expressed in human pancreatic carcinoma versus normal tissue. (A) Immunohistochemical staining for Polo-like kinase 1(brown) and counterstaining with hematoxylin illustrates Plk1 is over-expressed in the pancreatic cancer. Single cells positive for Plk1 are shown in high magnification. N: Normal pancreatic tissue; T: pancreatic cancer tissues. (B) Statistical analysis of immunohistological staining for Plk1, including 59 normal tissues and 88 pancreatic cancers(*,$P = .022$). Error bars represent standard deviations. (C) The expression of Plk1 is higher with an increased pathologic grade.

are correlation with the deregulation of PI3K/Akt pathway [20,33]. Therefore, we explore the influence of PI3K/Akt pathway on resistance of PC-chemotherapy. We got normal pancreas tissues, PC tissues and PC tissues after the patient accepted gemcitabine treatment, then we used IHC to detect the Akt level in three tissues. The data shows the Akt is over-expressed in PC tissues than normal tissues, at the same time, Akt level is higher in PC tissues after the patient appeared gemcitabine-resistance (Figure 3A), which suggested that the abnormal activation of PI3K/Akt pathway might be associated with chemoresistance in PC. Next, we used cellular experiment to validate our hypothesis. Cell proliferation influenced by different concentrations of gemcitabine was detected by CCK-8 assay (Figure 3B). The optimal concentrations of gemcitabine for different PC cells were IC50AsPC-1 = 15uM, IC50BxPC-3 = 7.5uM and IC50PANC-1 = 12.5uM, respectively. Then, we disposed PC cells with gemcitabine or LY294002 (PI3K/Akt inhibitor) or both combined. And the qRT-PCR and Western blotting showed gemcitabine could activate PI3K/Akt pathway

through up-regulating Akt and p-Akt level. While LY294002 had no effect on mRNA level of Akt, and protein level of p-Akt was down-regulated, which imply that LY294002 influenced PI3K/Akt pathway through decreasing phosphorylation of Akt (Figure 3, C and D). After the PC cells were treated 72 h, we examined the cell apoptosis of each group by flow cytometry. Compared to the control group, gemcitabine group (Gem), and LY294002 group (LY), using LY294002 combined gemcitabine (Gem+LY) could induce the highest apoptosis rate ($P < .001$) (Figure 3, E and F). Hence, the

Table 2. Expression of Plk1 in pancreatic cancer was detected by immunohistochemical staining and illustrates the expression of Plk1 is higher with an increased pathologic grade ($P = .019$) while there is no significant difference among gender, age and clinical stage ($P > .05$)

Expression of Plk1 in Pancreatic cancer				
Characteristics	n	Low expression	High expression	P
Gender				.173
M	47	34 (72%)	13 (28%)	
F	41	24 (59%)	17 (41%)	
Age				.728
<60	55	37 (67%)	18 (33%)	
≥60	33	21 (64%)	12 (36%)	
Pathologic grade				.019
1–2	61	45 (74%)	16 (26%)	
3	27	13 (48%)	14 (52%)	
Clinical Stage				.719
I-II	75	50 (67%)	25 (33%)	
III-IV	13	8 (62%)	5 (38%)	

Table 1. Expression of Plk1 in pancreatic tissue was detected by immunohistochemical staining and illustrates Plk1 is over-expressed in the pancreatic cancer

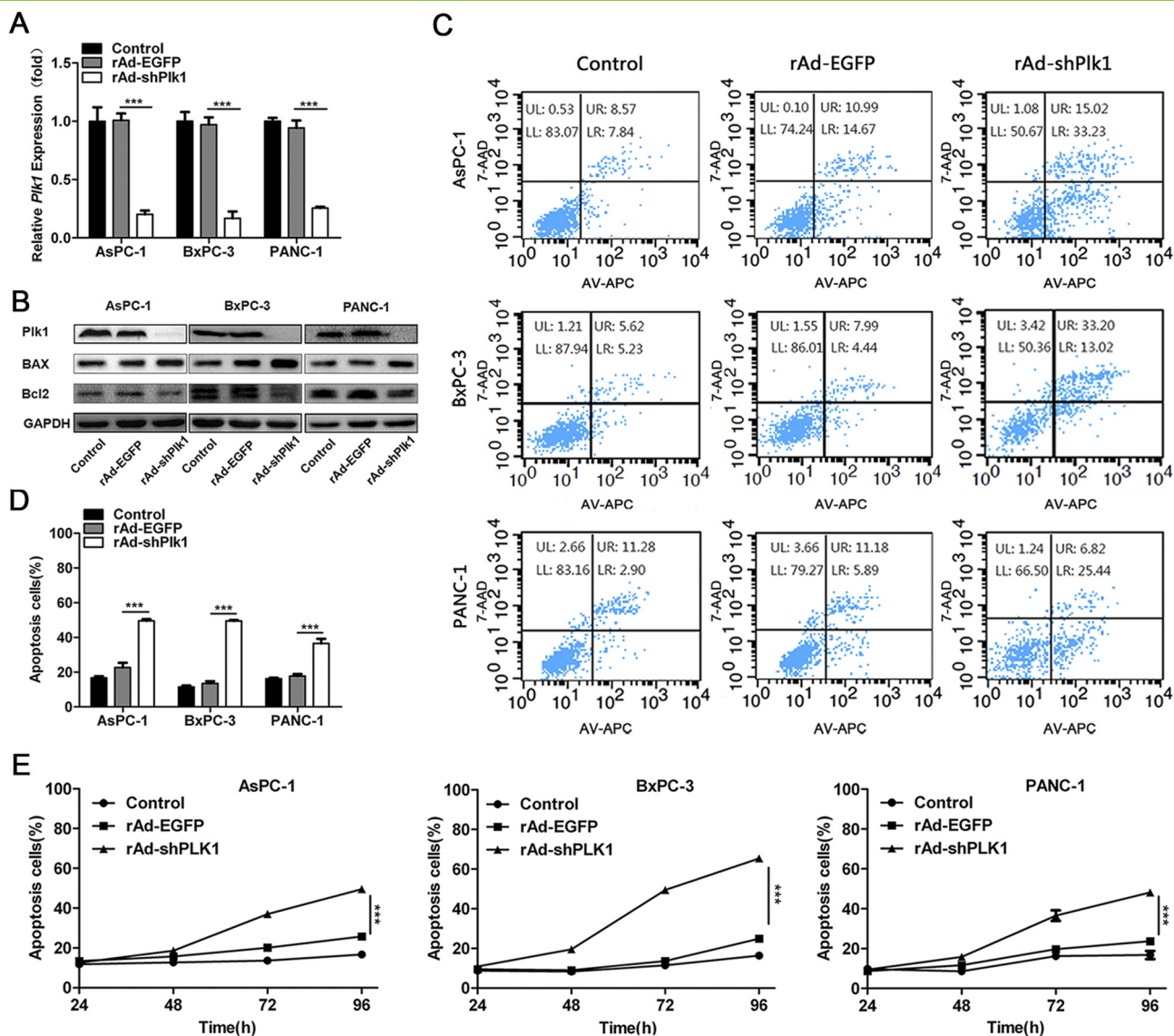| Expression of Plk1 in pancreatic tissue				
Group	n	Low expression	High expression	P
Normal tissue	59	49 (83%)	10 (17%)	.022
Pancreatic cancer	88	58 (66%)	30 (34%)	

Figure 2. Targeted depletion of Plk1 via recombinant adenoviral shRNA causes apoptosis in PC cells. (A) QRT-PCR was performed to detect Plk1 mRNA level and reveals lowest Plk1 mRNA level in rAd-shPlk1 group. Control: no treatment; rAd-EGFP: adenoviral vector which carries enhanced green fluorescent protein (EGFP) alone; rAd-shPlk1: adenoviral vector which carries EGFP-Plk1-shRNA. (B) The Plk1 and apoptosis-related proteins Bcl-2 and Bax protein expression levels in human pancreatic cancer cell lines were shown by Western blotting analysis. GAPDH expression was used as internal controls. The Plk1-siRNA reduces the expression of the Plk1 protein expression significantly and more cell apoptosis is observed in the rAd-shPlk1 group. (C) Pancreatic cancer cell lines were treated with recombinant adenovirus and harvested at 72 h. Apoptosis in different groups after infection was detected by Flow Cytometry. (D) Statistical analysis of apoptosis rate detected by Flow Cytometry at 72 h after recombinant adenovirus infection shows that the rAd-shPlk1 group cell apoptosis rate is significantly higher than that of the other two groups ($P < .001$). (E) Cell apoptosis rate detected by Flow Cytometry at 24 h, 48 h 72 h and 96 h after recombinant adenovirus infection revealsrAd-shPlk1 has a long-term apoptosis-inducing effect on pancreatic cancer cells and it works after 24 h post-infection. All the experiments were performed in triplicates. Error bars represent standard deviations.

results confirmed our previous hypothesis: the gemcitabine resistance to PC may be associated with abnormal activation of PI3K/Akt pathway, and LY294002 could increase chemosensitivity of PC cells to gemcitabine.

LY294002 Promotes Chemosensitivity of PC Through Downstream Plk1 Inactivation

Next, we ask did LY294002 increase chemosensitivity of PC cells to gemcitabine? It is reported that PI3K/Akt signal pathway are

closely related to resistance to apoptosis signal transduction and chemoresistance of PC [34,35]. On the other hand, Plk1 is essential for cell cycle, our previous study showed Plk1 is correlated to cell proliferation and chemoresistance in PC, and PI3K/Akt-dependent phosphorylation of Plk1-Ser99 is required for metaphase-anaphase transition [27,29]. So, it is possible that PI3K/Akt and Plk1 have interaction role in chemoresistance of PC. In order to test this point, we disposed PC cells with gemcitabine or LY294002 (PI3K/Akt inhibitor) or both combined and detected the Plk1mRNA and

protein level. As shown in Figure 4, C and D, the Plk1 expression level in PC cells with gemcitabine treatment showed up-regulated compared to control cells. While Plk1 expression level in LY294002 treatment showed down-regulated compared to control group, partly reversal gemcitabine up-regulating Plk1. And gemcitabine combined LY294002 could markedly up-regulating BAX and down-regulating Bcl-2, showed both combined can increase cell apoptosis (Figure 4, A and B). To further explore Plk1 in the process, we used rAd-shPlk1 to infect PC cells. The qRT-PCR and Western blotting showed Plk1 suppression has no influence on Akt and p-Akt (Figure 4, C and D), and as Figure 2 showed suppression Plk1 can induce PC cells apoptosis. Thus as a conclusion, we believed that LY294002 promotes chemosensitivity of PC to gemcitabine through down-regulating Plk1.

Targeted Depletion of Plk1 Enhances Chemosensitivity Induced by PI3K Inhibitor LY294002 in PC

Based on the above research, we reasoned that PI3K/Akt and Plk1 are both essential for chemoresistance in PC, and Plk1 as the downstream of PI3K/Akt regulate the chemosensitivity of PC. Hence, we further explored inhibition of PI3K/Akt and depletion of Plk1 on the effect of PC chemosensitivity. We treated PC cells with gemcitabine or LY294002 or rAd-shPlk1 or rAd-shPlk1 + Gem+LY. QRT-PCR and Western blotting showed rAd-shPlk1 + LY can reverse the role of gemcitabine up-regulating Plk1 obviously, and rAd-shPlk1 + Gem+LY can up-regulating BAX and down-regulating Bcl-2 significantly (Figure 5, A and B). Flow cytometry showed rAd-shPlk1 + Gem+LY could induce significant apoptosis in PC cells than the other groups ($P < .001$) (Figure 5, C and D). As a result, we think that the targeted depletion of Plk1 could enhance chemosensitivity induced by LY294002 in PC.

Inhibition of PI3K/Akt Signal Pathway and Down-regulation of Plk1 Combined with Gemcitabine Inhibit the Growth of PC Xenografts in Nude Mice

Finally, we investigated the effect of PI3K/Akt, Plk1 and gemcitabine in vivo through tumor xenograft model. We treated PC xenografts in nude mice with gemcitabine or LY294002 or BI2536(Plk1 inhibitor) or Gem+LY + BI2536. As shown in Figure 6, A–C, the tumor volume and the weight of Gem+LY + BI2536 group were significantly less than the other groups. Hematoxylin–eosin and IHC staining showed more necrosis and higher expression of Cleaved caspase-3 in Gem+LY + BI2536 group (Figure 6E). Meanwhile, Western blotting and TUNEL assay revealed Gem+LY + BI2536 induced PC cells apoptosis in the tumor xenograft model (Figure 6, D and E). Therefore, all the data above indicated that inhibition of PI3K/Akt signal pathway and down-regulation of Plk1 combined with gemcitabine could suppress PC growth and induced tumor necrosis and cell apoptosis in vivo.

Discussion

PC is one of the cancers with the highest mortality due to its difficulty in detection at early stage and lack of effective treatment at middle and late stage [36]. Although being the first-line chemotherapy drug for PC, gemcitabine still has some disadvantages, such as low drug sensitivity and significant side effects. Thus, how to further improve the sensitivity of PC cells to gemcitabine still focuses and difficult subject in the field of PC-treatment. In

recent years, deeply investigating the relationship between the key regulatory proteins of the DNA damage checkpoints and the signal pathways has become an import part in reversing the chemoresistance of tumors.

Plk1, as a crucial role in the regulation of cell proliferation, is up-regulated in the majority of PC cell lines and other human tumors [37]. Many studies have proved that even tumor metastasis and patients' survival are correlated with its overexpression [38–40]. Virginie et al. found Plk1 is overexpressed in triple-negative breast cancer and high Plk1 expression is associated with poor prognosis within the entire population, Plk1 is a potential therapeutic option combine with chemotherapy for triple-negative breast cancer patients [41]. On the other hand, many chemotherapeutic drugs via p53-dependent cell cycle and apoptosis act, and Plk1 could inactivate of p53, then Plk1 contribute to chemoresistance [13,42]. Our study showed Plk1 is overexpressed in PC tissues compare to normal tissues, and Plk1 expression level was correlated with pathologic grade, there were more PC tissues with Plk1 high expression in Pathologic grade III-IV than those in Pathologic grade I-II. Also, Weichert et al. observed that Plk1 is overexpressed in high-grade PanIN, the most important precursor lesion of PC and suggested that Plk1 overexpression is an early event in pancreatic cancer [14]. Therefore, it is not hard to imagine that Plk1 is associated with PC formation and progression. Then we constructed rAd-shPlk1, and found that depletion of Plk1 causes apoptosis in PC cells. Our earlier research showed that the Plk1 overexpression promoted cell proliferation, as well as an increase in G1/S phase cell percentage but a reduction in G2/M phase cell population [29]. Therefore, Plk1 is a potential therapeutic target for PC.

It is reported PI3K/Akt signal pathway controls G1 phase progression of PC cells regulating p21, SKP2, cyclin A, CDK2 etc. and after inhibition PI3K/Akt, SKP2 shows the most down-regulation, and PI3K/Akt inhibitor, LY294002, reduces Plk1 dephosphorylation following mitotic DNA damaging treatments, which suggesting that the PI3K pathway maybe involved in regulating Plk1 activity [41,43]. Schlieman et al. found activation of Akt existed in half of PC cases [44]. Our present study found Akt level is the highest in PC tissues after the patient appeared gemcitabine-resistance, so the gemcitabine resistance to PC may be associated with abnormal activation of PI3K/Akt pathway, and LY294002 could increase chemosensitivity of PC cells to gemcitabine.

Both the PI3K/Akt pathway and Plk1 play a major role in regulating the chemoresistance of PC at the level of DNA damage checkpoint. Zhang et al. reported Plk1 acts upstream of the PI3K/Akt/mTOR pathway during oxidative stress [32]. However, in cycle, PI3K/Akt-dependent phosphorylation of Plk1-Ser99 is required for metaphase-anaphase transition [27]. During our study we observed PI3K/Akt inhibition promotes chemosensitivity of PC through downstream Plk1 inactivation. Liu et al. HEATR1 regulates Akt phosphorylation at Thr308 by promoting Akt-PP2AB56β interaction, then regulates chemotherapy [45]. Our previous study showed suppress Plk1 expression can induce cancer cell apoptosis through activating caspases pathway and decreasing Bcl-2/BAX ratio [46].

Next we aims to investigate the influence of PI3K/Akt pathway combine Plk1 on sensitization of PC-chemotherapy, and it represent
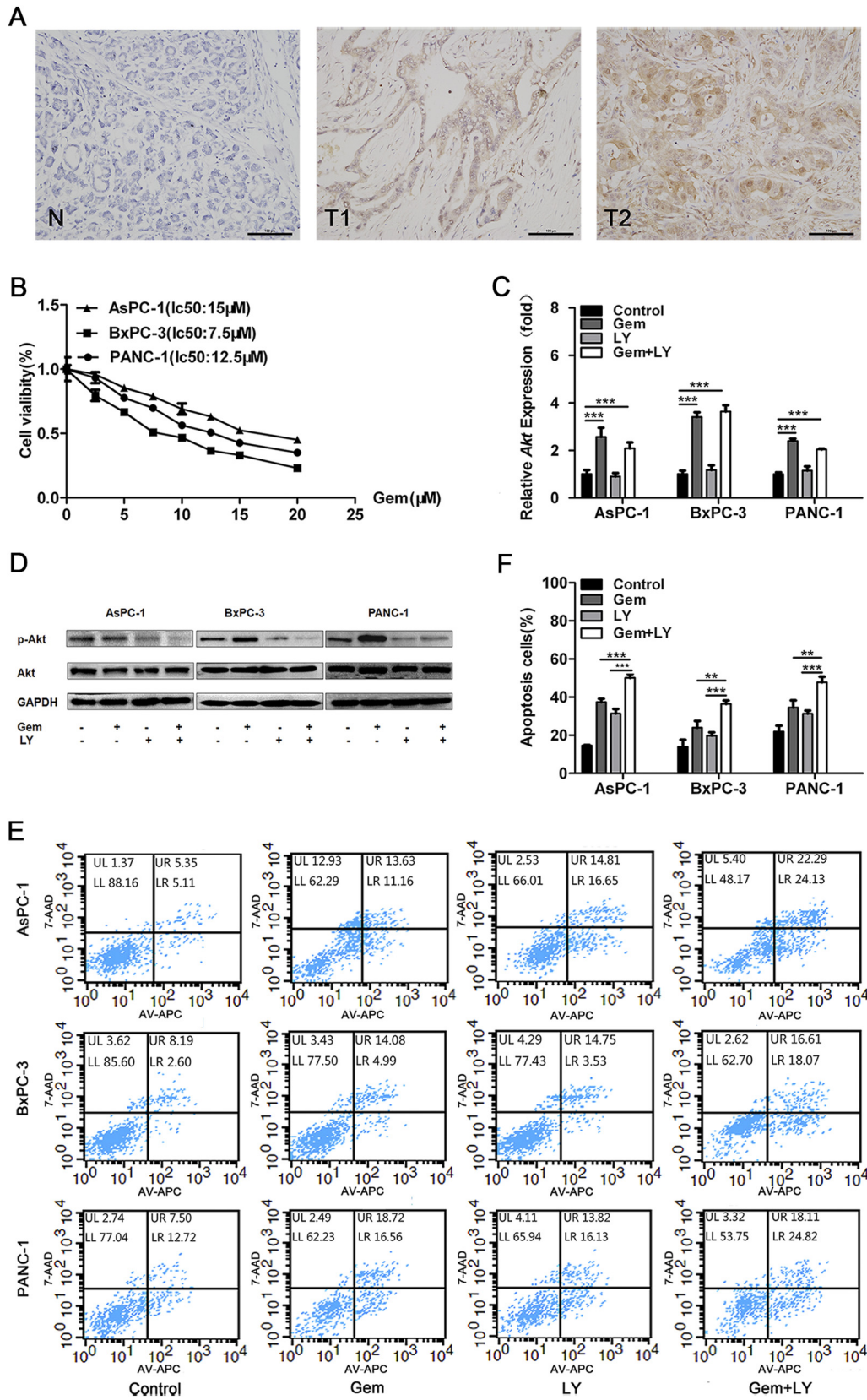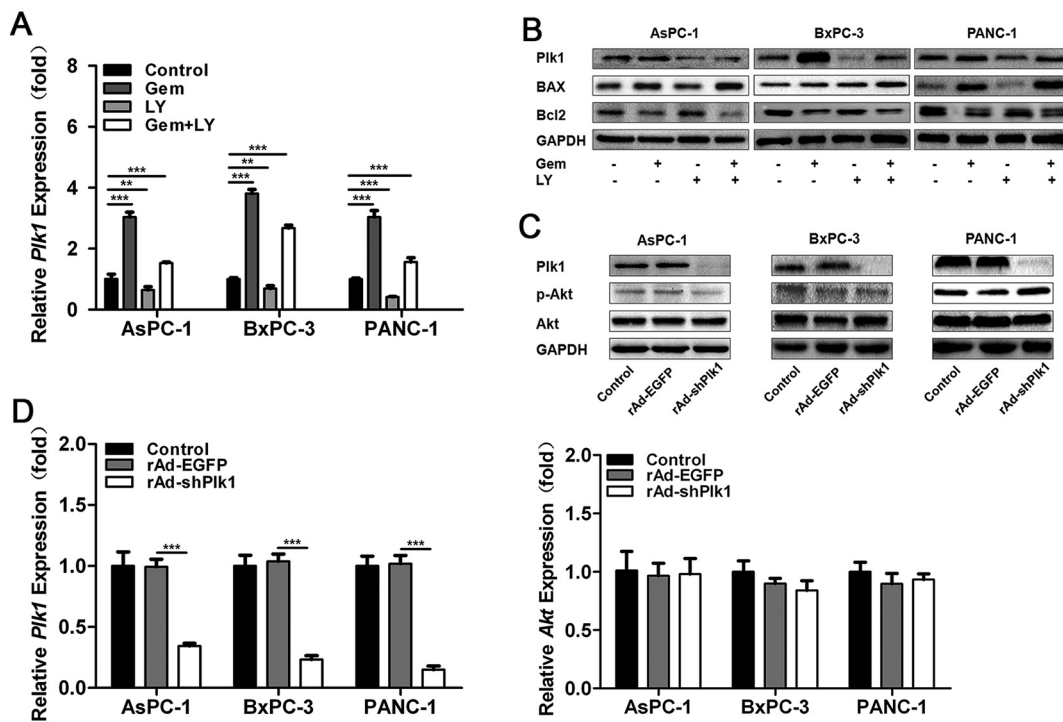
Figure 4. PI3K/Akt signal pathway promotes chemoresistance of PC through downstream Plk1 activation. (A) Plk1 mRNA level detected by qRT-PCR illustrates gemcitabine up-regulates the Plk1 expression in pancreatic cancer ($P < .001$) while PI3K inhibitor LY294002 can reverse this process ($P < .001$). (B) The Plk1 and apoptosis-related proteins Bcl-2 and Bax protein expression levels in human pancreatic cancer cell lines were shown by Western blotting analysis. GAPDH expression was used as internal controls.PI3K inhibitor LY294002 reduces Plk1 expression and can lower the effect of Plk1 up-regulating by gemcitabine. Combination of gemcitabine and LY294002 enhances the cytotoxicity of gemcitabine. (C) The Plk1 and Akt protein expression levels in human pancreatic cancer cell lines were detected by Western blotting analysis. GAPDH expression was used as internal controls. Plk1 knockout through shRNA shows no influence in Akt expression. (D) QRT-PCR illustrates the same result as that showed in the previous Western Blotting analysis and proves Plk1-shRNA does not influence the Akt expression.

suppression of Plk1 could enhance chemosensitivity induced by inhibition of PI3K/Akt in PC cells in vitro. At last, we verify our hypothesis in vivo, we used LY294002 (PI3K inhibitor), BI2536 (Plk1) inhibitor or gemcitabine treat PC xenografts nude mice, and the result shows inhibition of PI3K/Akt and down-regulation of Plk1 combined with gemcitabine inhibit the growth of PC xenografts in nude mice. However, a phase II/III randomized study showed the combination of rigosertib(a first-in-class Ras mimetic and small-molecule inhibitor of multiple signaling pathways including Plk1 and PI3K) + gemcitabine failed to demonstrate an improvement in survival or response compared with gemcitabine in patients with metastatic PC [47]. Both LY294002 and BI2536 phase I trials revealed that the drug was well tolerated with minor antitumor responses, and they also can enhance chemosensitivity to gemcitabine in PC cells [18,48]. In summary, our findings confirm that suppression of PI3K/Akt and Plk1 combined gemcitabine could be a potential therapeutic schedule for PC patients, and further efforts need to be done.

Disclosure of Interest

The authors declare that they have no conflicts of interest concerning this article.

Figure 3. Gemcitabine-resistance in PC is closely related to PI3K/Akt signal pathway. (A) Immunohistochemical staining for Akt(brown) and counterstaining with hematoxylin illustrates Akt is over-expressed in the pancreatic cancer. At the meantime, Akt expression is higher in the pancreatic cancer tissue after the patient accepted gemcitabine treatment. N: Normal pancreatic tissue; T1: pancreatic cancer tissue without gemcitabine treatment; T2: pancreatic cancer tissue with gemcitabine treatment. (B) MTT assay was performed to detect cytotoxicity of gemcitabine on pancreatic cancer cell lines and the appropriate concentrations of Gemcitabine in different cell lines are $IC50_{AsPC-1} = 15uM$, $IC50_{BxPC-3} = 7.5uM$ and $IC50_{PANC-1} = 12.5uM$. The relative cellular viability was measured by setting untreated cells as 100% cell viability. (C) Akt mRNA level tested by qRT-PCR illustrates gemcitabine up-regulates the Akt expression in pancreatic cancer ($P < .001$) while PI3K inhibitor LY294002 can reverse this process ($P < .001$). (D) Akt protein level tested by Western Blotting shows the same results as that of qRT-PCR. (E) Pancreatic cancer cell apoptosis was detected by Flow Cytometry at 72 h after gemcitabine or LY294002 or both drugs treatment. (F) Statistical analysis of apoptosis rate detected by Flow Cytometry at 72 h after drugs treatment reveals combination of gemcitabine and LY294002 enhances chemosensitivity of gemcitabine on pancreatic cancer cells ($P < .001$). All the experiments were performed in triplicates. Error bars represent standard deviations.
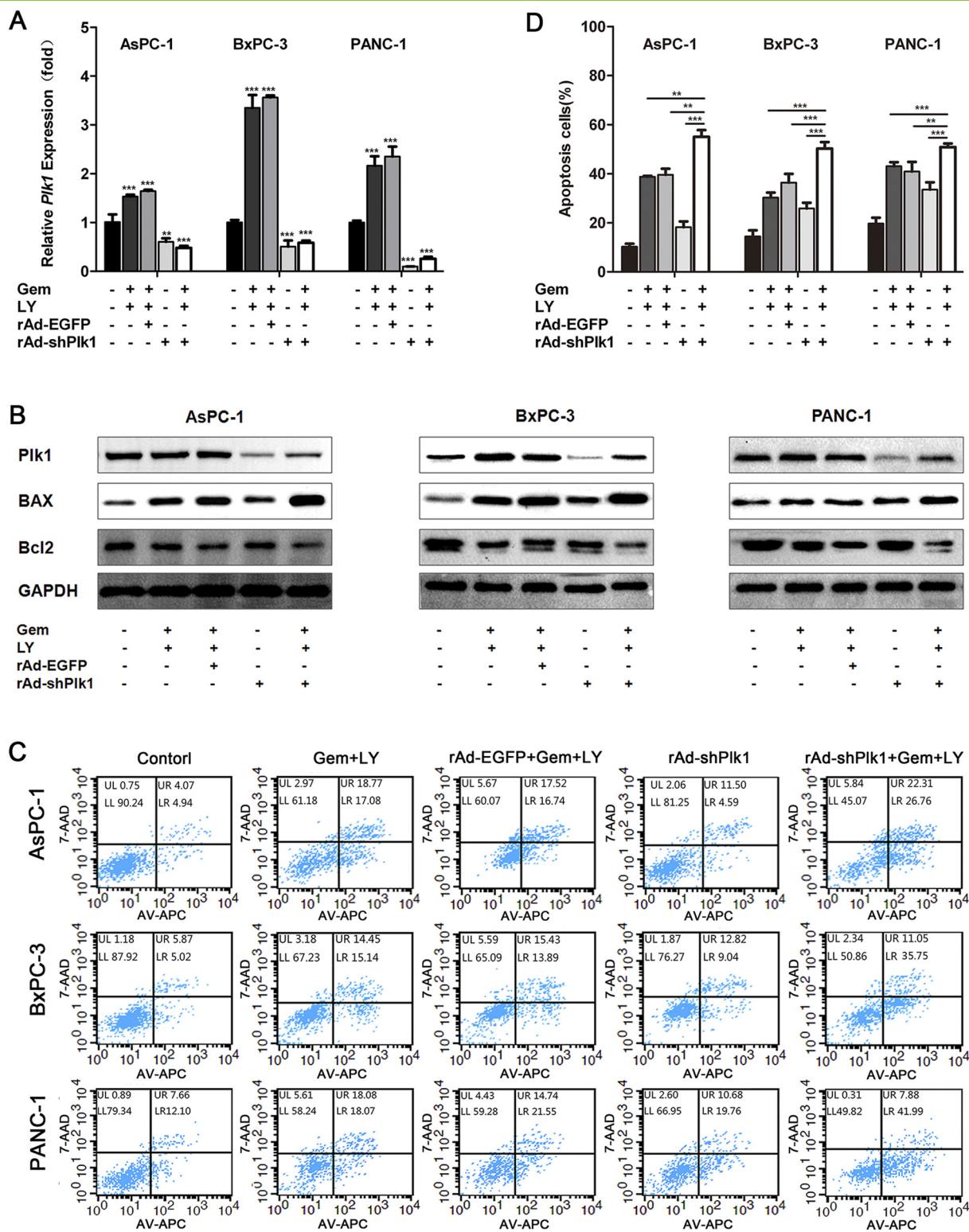
Figure 5. Targeted depletion of Plk1 enhances chemosensitivity induced by PI3K inhibitor LY294002 in PC. (A) The Plk1 mRNA level detected by qRT-PCR shows Plk1 knockout significantly reduces the effect of Plk1 up regulating by gemcitabine in pancreatic cancer cell lines ($P < .001$). (B) The Plk1 and apoptosis-related proteins Bcl-2 and Bax protein expression levels in human pancreatic cancer cell lines were shown by Western blotting analysis. GAPDH expression was used as internal controls. The Plk1 protein level under the same conditions is similar as its mRNA level. The Bcl-2 and Bax protein levels reveal that Plk1 knockout through shRNA enhances the chemosensitivity of combined chemotherapy and results in more cell apoptosis. (C) Pancreatic cancer cell apoptosis was detected by Flow Cytometry. Apoptosis rate in rAd-shPlk1 + Gem+LY group is higher than it in other four groups. (D) Statistical analysis of apoptosis rate detected by previous Flow Cytometry shows targeted depletion of Plk1 enhances chemosensitivity induced by PI3K inhibitor LY294002 in pancreatic cancer and results in higher cell apoptosis rate ($P < .01$).
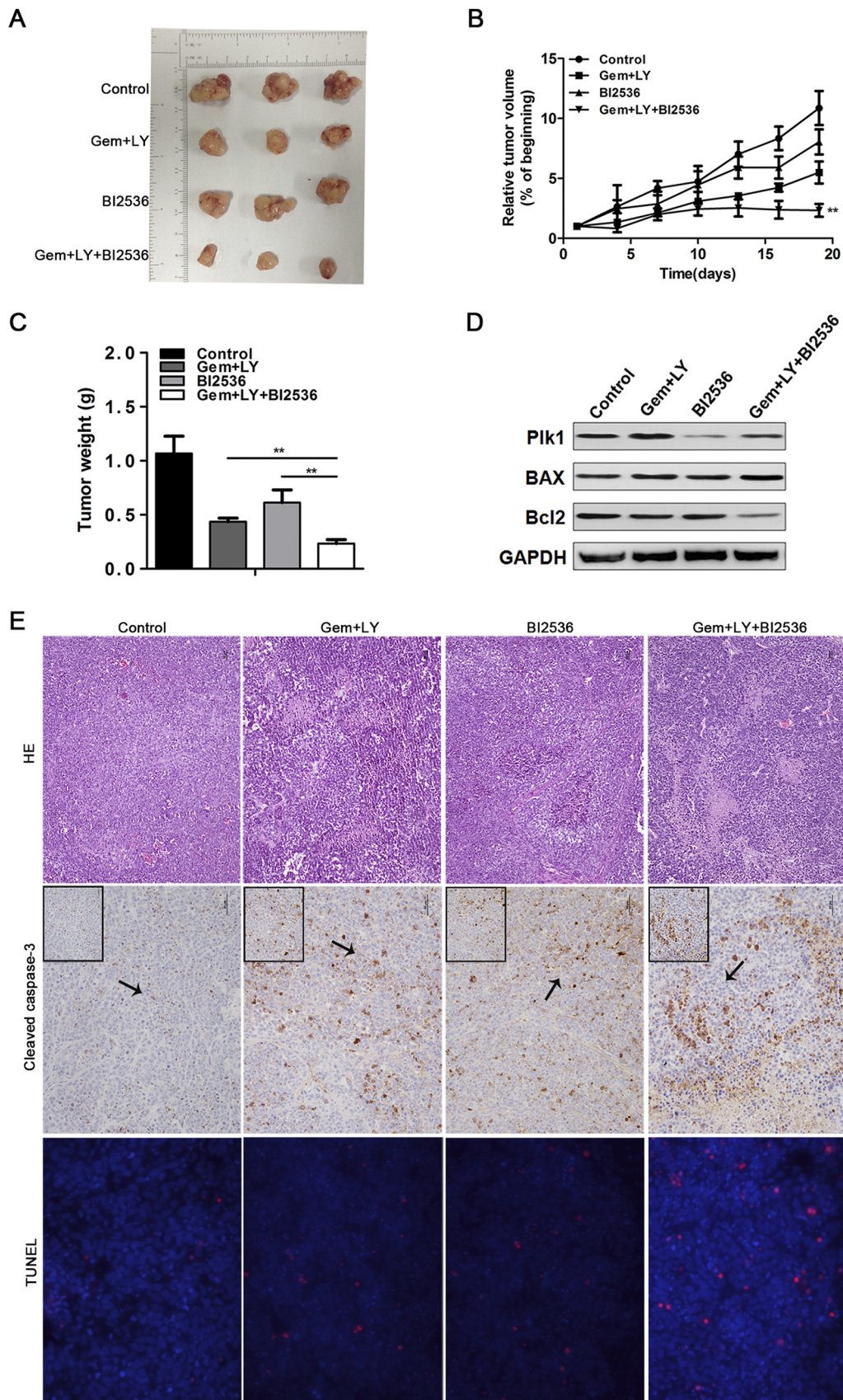
Figure 6. Inhibition of PI3K/Akt signal pathway and down-regulation of Plk1 combined with gemcitabine inhibit the growth of PC xenografts in nude mice. (A, B and C) Gem+LY + BI2536 could diminish the tumor size and weight according to measurement of the resected tumors(, *P* < .01). (D and E) Western blotting and Hematoxylin–eosin staining showed more necrosis and the highest expression of Cleaved caspase-3 in Gem+LY + BI2536 group. TUNEL assay showed the highest cell apoptosis rate in Gem+LY + BI2536 group than that in the control group, Gem+LY group and BI2536 group.

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